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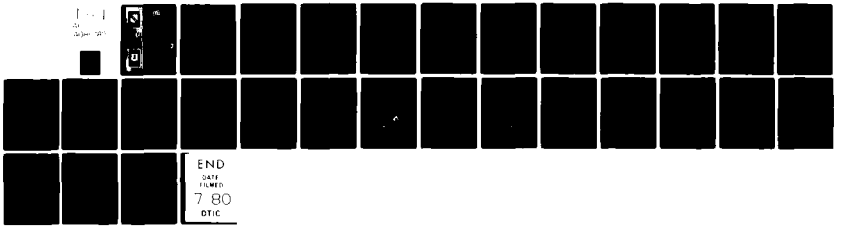

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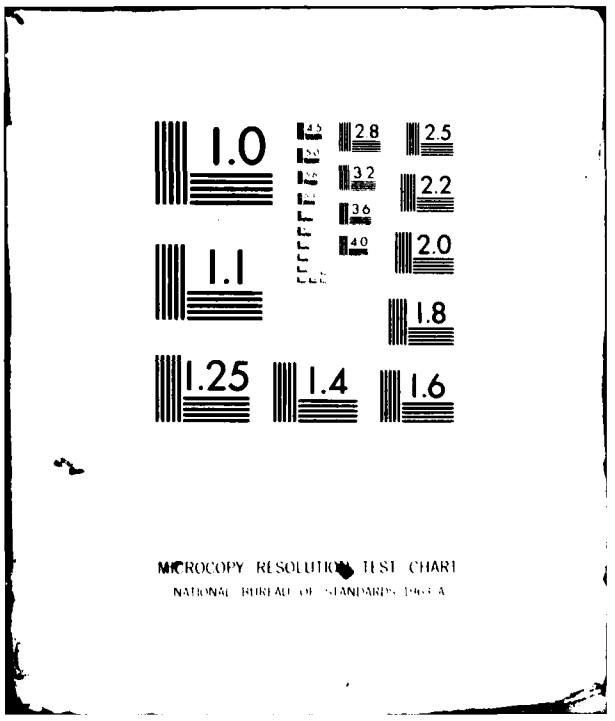
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FRANK J. SEILER RESEARCH LABORATORY

FJSRL TECHNICAL REPORT 80-004
FEBRUARY 1980

THE EFFECT OF HYPERBARIC OXYGEN ON THE GROWTH OF MUCOR SP. AND ASPERGILLUS FUMIGATUS

By

WILLIAM J. CAIRNEY

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PROJECT 2303

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This technical report has been reviewed and is approved for publication.

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Mucormycosis	Aspergillosis	Mycotic Disease Agents
Mucor	Hyperbaric Medicine	Oxygen Pressure
Aspergillus fumigatus	Hyperbaric Oxygenation	Oxygen Therapy
Compression Chamber	Hyperbarics	
Compression Therapy	Medical Mycology	

20. ABSTRACT (Continue on reverse side if necessary and identify by block number)

Mucor sp. and Aspergillus fumigatus isolated from different human lung infections were observed in vitro for macroscopic and microscopic growth characteristics at varying hyperbaric oxygen levels and for various exposure times and intervals. Both organisms were completely inhibited in oxygen at 3 atmospheres absolute and were at least retarded in all growth processes when subjected to hyperbaric oxygen according to exposure tables routinely used for treating Clostridial gas gangrene. Overall experimental results provide in vitro basis for use of compression chamber therapy in treatment of

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Mucormycosis and Aspergillosis.

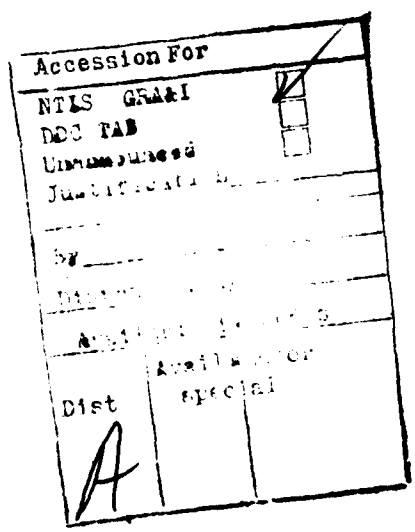
FJSRL-TR-80-004

THE EFFECT OF HYPERBARIC OXYGEN ON THE GROWTH OF *MUCOR* SP. AND *ASPERGILLUS FUMIGATUS*

By

Major William J. Cairney

February 1980



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INTRODUCTION

Infections caused by anaerobic organisms such as Clostridium spp. have been successfully treated with hyperbaric oxygen for several years. Pseudomonas infections secondary to burns have been treated also, often with excellent results. Pseudomonas spp. are not considered anaerobic but apparently have oxygen tolerance limits low enough to be inhibited by levels easily tolerated by human patients. Even though cases have been limited in number, empirical evidence exists that hyperbaric oxygen treatment is also effective against human fungal infections. Hyperbaric oxygen has been used as a last resort treatment in at least one case each of mycoses caused by Actinomyces sp., Aspergillus sp., Mucor sp., and Coccidioides immitis. In some cases Amphotericin B was used in conjunction with hyperbaric oxygen, in other cases not, but in each case the mycosis cleared. In one case, a patient with "malignant mucormycosis" involving muscle tissue was treated with hyperbaric oxygen. The patient also had a staphylococcal septicemia and malaria. All of these processes were reported cleared following treatment (4).

To date, only a few papers report systematic in vitro studies of the effects of hyperbaric oxygen on funal organisms. Gottlieb has compliled an excellent review of these papers and indicates in table form which organisms have been tested (5). Inspection of the actual papers reveals several dilemmas for the would-be researcher. Dilemmas include a) significant variability and contradictions in available data; b) possible erroneous correlations (or non-correlations) of susceptibility of certain fungal taxa to inhibition by increased oxygen levels; c) questionable reliability of results of past work in terms of possible misidentification of organisms used; d) nomenclatural controversies

involving major human pathogenic fungal groups; and, e) questionable reproducibility of certain studies due to tendencies of human pathogenic fungi to change with time and conditions. Cairney has detailed these problems and has suggested how they may be avoided in future work (2).

Both Mucor spp. and Aspergillus spp. have been investigated already relative to their growth or lack of growth in hyperbaric oxygen. All studies, however, have been exploratory and have centered around inhibition of growth in culture (5). For the most part, organisms have been reported as having lived or died given various doses of oxygen. No observations have been made correlating cytological features with macroscopic growth patterns.

The purpose of this study was to determine growth rates and observe macroscopic and microscopic appearance of Mucor sp. and Aspergillus fumigatus (both isolated from human lung) under various hyperbaric oxygen levels.

MATERIALS AND METHODS

Five experiments were undertaken to determine the effects of hyperbaric oxygen on these organisms. Isolates of both fungi were obtained from the Armed Forces Institute of Pathology. Following experiments to determine media for most observable and repeatable growth characteristics (2), both organisms were maintained on Sabouraud's dextrose agar in tissue culture flasks. Sabouraud's agar was also used for all single spore preparation and growing of fungi exposed to hyperbaric oxygen. All exposures of the organisms to increased oxygen pressures were carried out in a table top (31 liter capacity) hyperbaric chamber made available for the project by the Aerospace Pathology Branch of the Armed Forces Institute of Pathology.

In all experiments, organisms were compressed to the desired pressure in less than 30 seconds. Following compression, the chamber was flushed rapidly with five chamber volumes of 100% oxygen to insure a pure oxygen environment. Each time the chamber was decompressed for removal of cultures, this same compression/flushing operation was performed upon continuation of the experiment. During compression periods, a 5-liter/hour flow was maintained through the system.

Organisms to be exposed to oxygen, along with controls, were propagated from single spores poured in water suspensions on Petri plates containing Sabouraud's agar. Suspensions were poured 24 hours before transfer of plugs containing germinating single spores to Sabouraud's agar plates to be used for actual exposures. In all experiments, each plate contained four single spores of a given organism placed on the medium, one in the center of each quadrant. No inhibitors of any kind were incorporated into the medium in order to reduce variables. The pH of the medium was 5.6. The room in which the experiments were performed was held at 25°(±1°)C. Cultures were exposed to room fluorescent light for the duration of all experiments. The hyperbaric chamber, constructed of transparent plexiglas, allowed exposure of organisms to room light.

In the first experiment, single-spore cultures of Mucor sp. and Aspergillus fumigatus were placed in the hyperbaric chamber and exposed to a steady 3-ATA (3 atmospheres absolute) level of oxygen. Controls were allowed to grow in air under room pressure. Air pressurized controls (3-ATA) were run at a separate time but under otherwise identical conditions to insure that effects were caused by oxygen rather than total pressure. Four 1-ATA air control plates (total of 16 colonies) and four

air pressurized (3-ATA) control plates (16 colonies) were run for each experiment.

Organisms were removed from the chamber at intervals of 24, 48, 96, 120, 144, 168, 192, 216, 240, and 264 hours. At each interval, four plates (16 colonies) were removed and surveyed for macroscopic and microscopic growth features. Colony diameters were measured as an index of linear growth. All plates were retained for three weeks from initiation of the experiment and measurements made of colonies on each plate at the same time each day. Microscopic mounts were made to correlate microscopic development with macroscopic features.

In removing plates from the chamber for examination, the system was depressurized over a period of 30 seconds. Appropriate plates were removed and the chamber was repressurized in 30 seconds. The entire system was immediately flushed with 5 chamber volumes of oxygen to return the internal environment to correct total pressure and PO_2. Plates not to be removed were left undisturbed as much as possible, except that where growth was evident in the chamber, plates were removed quickly for measurement and then replaced. The entire depressurization-repressurization-flush procedure took no more than five minutes.

In a second experiment, Mucor sp. and Aspergillus fumigatus cultures were exposed to hyperbaric oxygen according to exposure tables used for treating gas gangrene resulting from Clostridium spp. (especially Clostridium perfringens) infections (1). Petri plates containing 24-hour, single-spore cultures of Mucor sp. and of A. fumigatus were prepared for chamber exposure for each organism. Four 1-ATA and four 3-ATA air controls were also run in a similar manner for each.

The experimental plates were exposed to 3-ATA oxygen for three

90-minute periods during the first 24 hours, treatments beginning approximately eight hours apart. In each of two succeeding 24-hour periods, cultures were exposed to the same 3-ATA level for two 90-minute periods, treatments beginning approximately 12 hours apart. Cultures were thus exposed to seven 90-minute periods of oxygen at 3-ATA over a span of three days. Organisms were checked for growth throughout the experiment and measurements were made where appropriate. Microscopic features were observed regularly. (Again, 3-ATA air controls were run using identical methods but at another time because only one hyperbaric chamber was available for use.)

Results from these first two experiments suggested that some effect might be realized using the same 3-ATA level but at longer intervals between exposures. Two additional experiments were thus performed. In the first, organisms were exposed to the 90-minute, 3-ATA level at 18-hour intervals. In the second, organisms were exposed at 12-hour intervals.

In a final experiment, Mucor sp. and A. fumigatus were exposed to 1-ATA oxygen to determine the effects of steady exposure to this lower PO_2. Plates were prepared in the same manner as for the 3-ATA experiment. Organisms were observed frequently over a 7-day period. Colonies were measured every 24 hours.

RESULTS

Growth curves for all organisms in the 3-ATA experiment are shown in figures 3 and 4. Growth curves for the "treatment table" exposures are shown in figures 5 and 6. Bars indicate upper and lower limits of readings. For the most part, those limits were not very great. Fungi exposed to identical conditions (whether on the same actual Petri plate

or not) grew to the same extent over a given time interval. Microscopic features of Mucor sp. and Aspergillus fumigatus are illustrated in figures 1 and 2 for controls. Comparison of exposed organisms made directly in the text. On all graphs, controls are marked "c". Exposed plates are marked "e". The number following "e" indicates the hour interval at which that set of plates was removed from the chamber.

Data from these experiments confirm the general results of some past studies and tends not to confirm results of others. McAllister et al. examined colony growth of A. fumigatus under hyperbaric oxygen and reported inhibition at 2-ATA (8). This is consistent with results in this study where A. fumigatus grew readily at 1-ATA, but failed to grow at all as long as the culture was exposed to 3-ATA.

Robb and Caldwell, in separate studies, investigated the effects of 10-ATA oxygen pressure on many fungi including Aspergillus spp. (3, 9). Robb and Caldwell were both interested in time tolerance limits at that pressure. While the results of the Robb and Caldwell research are not directly comparable to those in this study due to different pressures and other parameters, both reported a similar phenomenon in cultures removed from oxygen pressure. Both described a "lag" period in which no growth occurred. Following the "lag" period, growth (usually normal growth) resumed.

A "lag" period was also seen in these experiments, especially after exposure to 3-ATA oxygen. This author proposes that the "lag" period be defined as the time it takes for an exposed isolate, following removal from hyperbaric oxygen, to reach a given point on the growth curve, minus the time taken by the control to reach the same point. Thus, for Mucor sp. in the experiment, the control reached a 2 mm diameter by

hour 24. Plates exposed for 24 hours reached a 2 mm diameter by hour 53. Given the 24-hour period of no growth in the chamber and the 29 hours necessary to achieve a 2 mm diameter, the lag time would equal 5 hours.

This definition certainly has its limitations, but if a low but measurable growth curve point is selected, a reasonable approximation of lag time can be calculated. Unless one were to use Riddell slide cultures, expose them to hyperbaric oxygen, and observe hyphal tips constantly upon removal from the chamber, it would be very difficult to pinpoint the exact moment of resumption of growth. The definition breaks down if a growth curve point much higher than 5 mm is selected, because differences in growth curve slope (as seen in figure 3) will affect the calculation.

Lag times (as defined here) for A. fumigatus were very short and very consistent from interval to interval and from one repetition of the experiment to another. At 3-ATA the A. fumigatus lag time was 19 hours. Mucor sp. lag times were not as regular, but increased with exposure time. Organisms exposed for 24 hours had only a 5-hour lag. Organisms exposed for 48 hours lagged for 29 hours. From this point, lag times rose to 43 hours for the 72-hour exposures. 46 hours for the 96-hour exposures, and 43 hours for plates exposed for 120 hours.

From the points of growth resumption, all isolates of Mucor sp. grew vegetatively at a faster rate than controls. The progressive increase in growth curve slope is evident in figure 3. Approximate rate of radial growth of controls is calculated to be 0.16 mm/hour. For el20 organisms, radial growth was 0.45 mm/hour. A significant but opposite effect was seen at the microscopic level. All isolates exposed to 3-ATA oxygen were much slower in development of sporangia. 1-ATA and 3-ATA air controls had sporangia present by day 7. Organisms exposed to 3-ATA oxygen took at

least an additional three days relative to controls to produce these structures. When sporangia did appear, they seemed identical to those of controls. It appears that a recovery is realized in vegetative growth following removal from oxygen, but recovery in ability to sporulate is much slower.

This same inability to sporulate was observed in A. fumigatus also. Conidia were present in all controls by day 4 (figure 2). Exposed organisms again took about three additional days (relative to controls) to produce conidial heads. Thus, even though a given A. fumigatus colony may have resembled a 4-day control colony in size, it resembled a much younger control colony microscopically.

In the Robb, Caldwell, and McAllister papers, no such microscopic observations were made. In addition, no previous worker has every subjected these organisms to "treatment table" exposures.

Figures 5 and 6 show for both organisms exposed to the hyperbaric oxygen treatment table that fungal growth, while not inhibited altogether, is at least retarded. Once the fungus has begun to grow, there is no appreciable difference in the slope of the growth curve.

Exposing the organisms to 90-minute periods of 3-ATA oxygen at 18-hour and 12hour intervals produced no dramatic results. Vegetative growth of the controls and experimentals was so close that statistical analysis indicated that any differences were not significant. Microscopic features were the same from controls to exposed plates.

Exposure to 1-ATA oxygen likewise resulted in no significant differences in growth rates or microscopic features. In one of the earliest papers on hyperbaric oxygen and fungi, Karsner and Saphir in 1926 (6) examined the influence of elevated partial pressures of oxygen on the

growth of many saprophytic and pathogenic molds including one Aspergillus species (not A. fumigatus). These researchers used the same medium as used in this present study, exposing organisms to 50%-99% oxygen (at 1-ATA) for 2.5 to 8 days. Colony morphology was used as the comparison criterion. Karsner and Saphir reported that oxygen concentrations of 76% or greater had inhibitory effects on most molds in their study and that inhibition tended to be greater in pathogens than in non-pathogens. The data from the current study do not support Karsner's and Saphir's results.

DISCUSSION

The obvious intent of this investigation is to establish an in vitro cytological basis for treating cases of Aspergillosis and Mucormycosis in hyperbaric chambers. To some extent, that basis has been confirmed. The organisms are at least retarded by oxygen levels well tolerated by humans.

The rationale behind the 90-minute exposures at 18-hour and 12-hour intervals is that perhaps the fungi would be greatly inhibited if they were re-exposed within the lag periods calculated on the basis of the 3-ATA oxygen steady exposure experiment. It seems possible on the basis of results obtained that an exposure time well in excess of 90 minutes is necessary to establish a lag period. This is supported by the very short (5-hour) lag period of Mucor sp. after 24 hours at 3-ATA oxygen.

The 1-ATA oxygen exposure rate, had it confirmed the Karsner and Saphir work, would have at least suggested the possibility of treatment with 100% O_2 without the need for additional pressurization.

The demonstration of an effect using a standard treatment table is significant and the effect of the hyperbaric oxygen might be greatly enhanced with systemic antifungal agents used between oxygen exposures.

Recent unpublished data from this laboratory (10) suggest that some antifungal agents, including Amphotericin B, may actually be degraded by exposure to hyperbaric oxygen. One or more of the breakdown products may be metabolites. Should this be confirmed in future work, combining hyperbaric oxygen with systemic antifungal agents administered at the same time might not be advisable.

Follow-on studies testing these organisms *in vivo* would supply additional helpful information. The recent *Mycobacterium ulcerans* experiments by Krieg et al. would be a possible model for such work (7).

ACKNOWLEDGMENTS

The author gratefully acknowledges the support of Dr. Richard P. Korf, Professor of Mycology, Dr. Carl W. Boothroyd, Professor of Plant Pathology, and Dr. John Bentinck-Smith, Professor of Clinical Pathology, all of Cornell University, for critical review of the manuscript. The support of Dr. John H. Wolcott and Dr. Richard E. Krieg of the Armed Forces Institute of Pathology was invaluable for providing cultures and equipment. Portions of this paper represent part of a thesis presented to the Cornell University Graduate School for the degree of Doctor of Philosophy. An active program on hyperbarics and mycotic disease agents is presently on-going in the Department of Chemistry and Biological Sciences, United States Air Force Academy. The author appreciates the support of the Frank J. Seiler Research Laboratory in sponsoring this effort.

Note: Reference cultures (dried) of *Mucor* sp. and *Aspergillus fumigatus* are on file in the Plant Pathology Herbarium at Cornell University. Reference numbers are CUP 54846 and CUP 54845, respectively.

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Figure 1. Mucor sp.

Upper Frame - Coenocytic mycelium, day 3.

Lower Frame - Sporangiophores bearing sporangia, day 7.

Time designations are for controls.

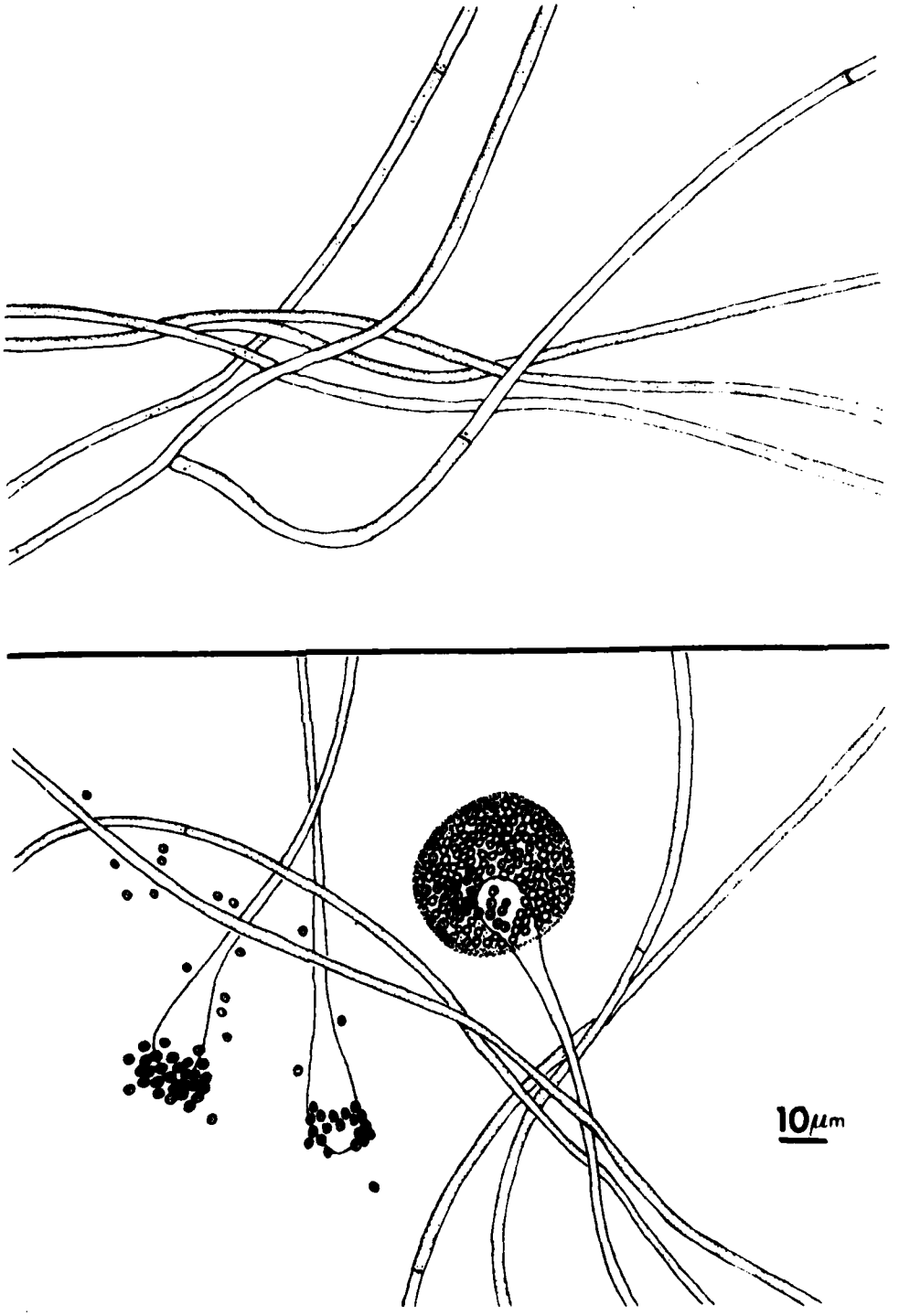
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Figure 2. Aspergillus fumigatus

Top Frame - Hyphae at day 2.

Middle Frame - Hyphae with developing conidiophores, day 3.

Lower Frame - Hyphae with conidiophores and conidia, day 4.

Time designations are for controls.

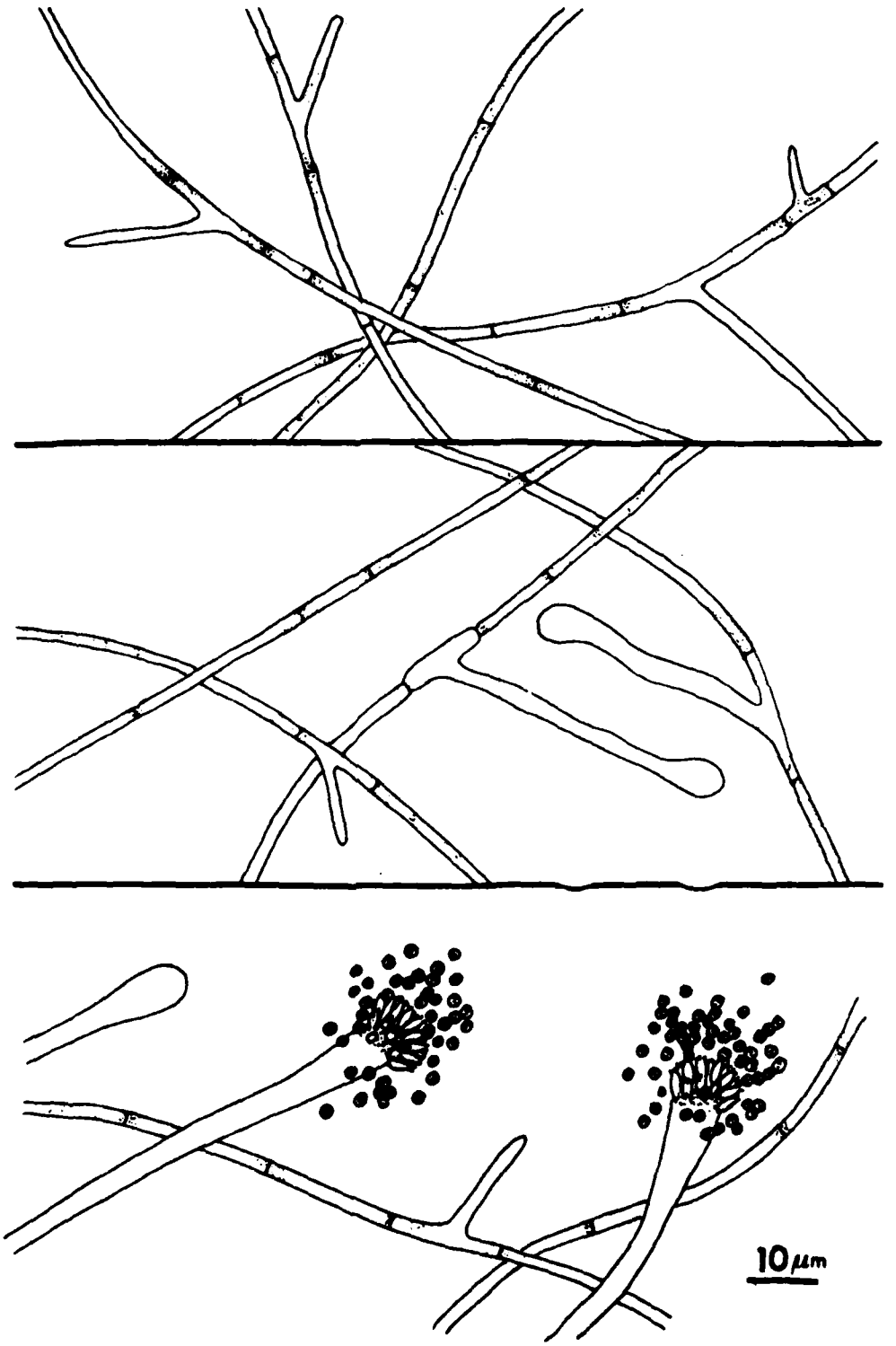
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Figure 3. Growth curves for cultures of Mucor sp. exposed to 3-ATA oxygen for indicated intervals.

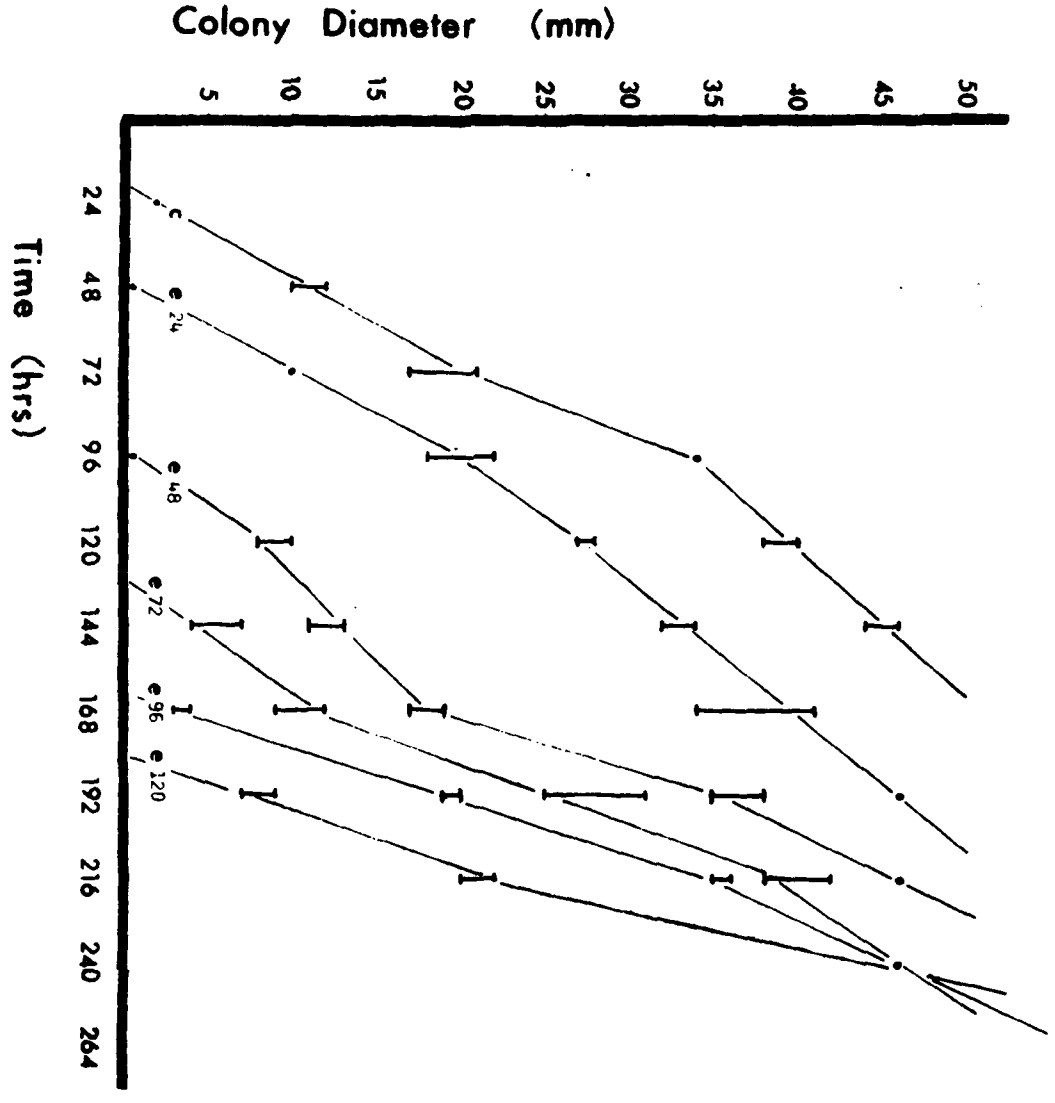
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Figure 4. Growth curves for cultures of Aspergillus fumigatus exposed to 3-ATA oxygen for indicated intervals.

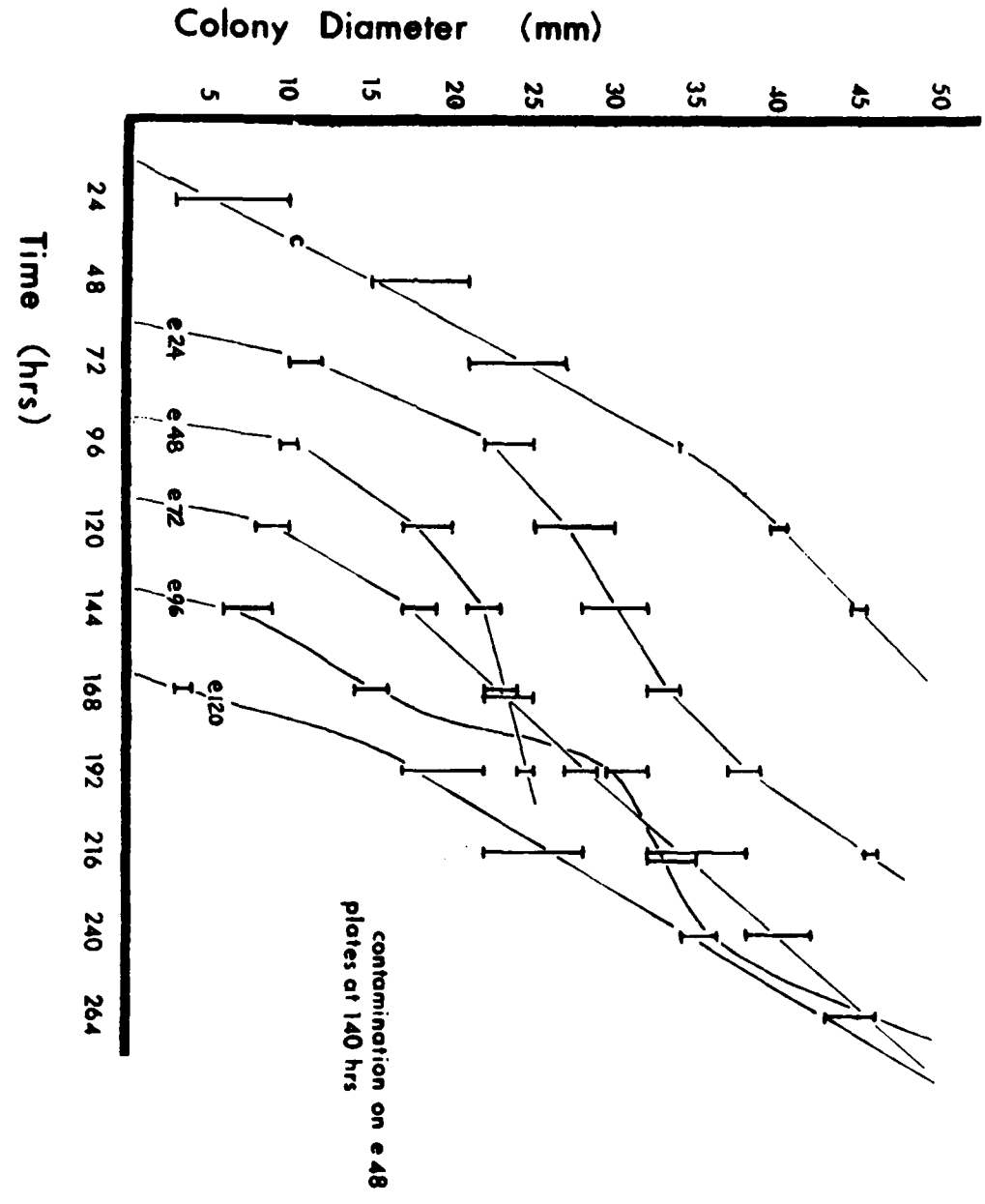
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Figure 5. Growth curves for cultures of Mucor sp. subjected to "treatment table" oxygen exposures.

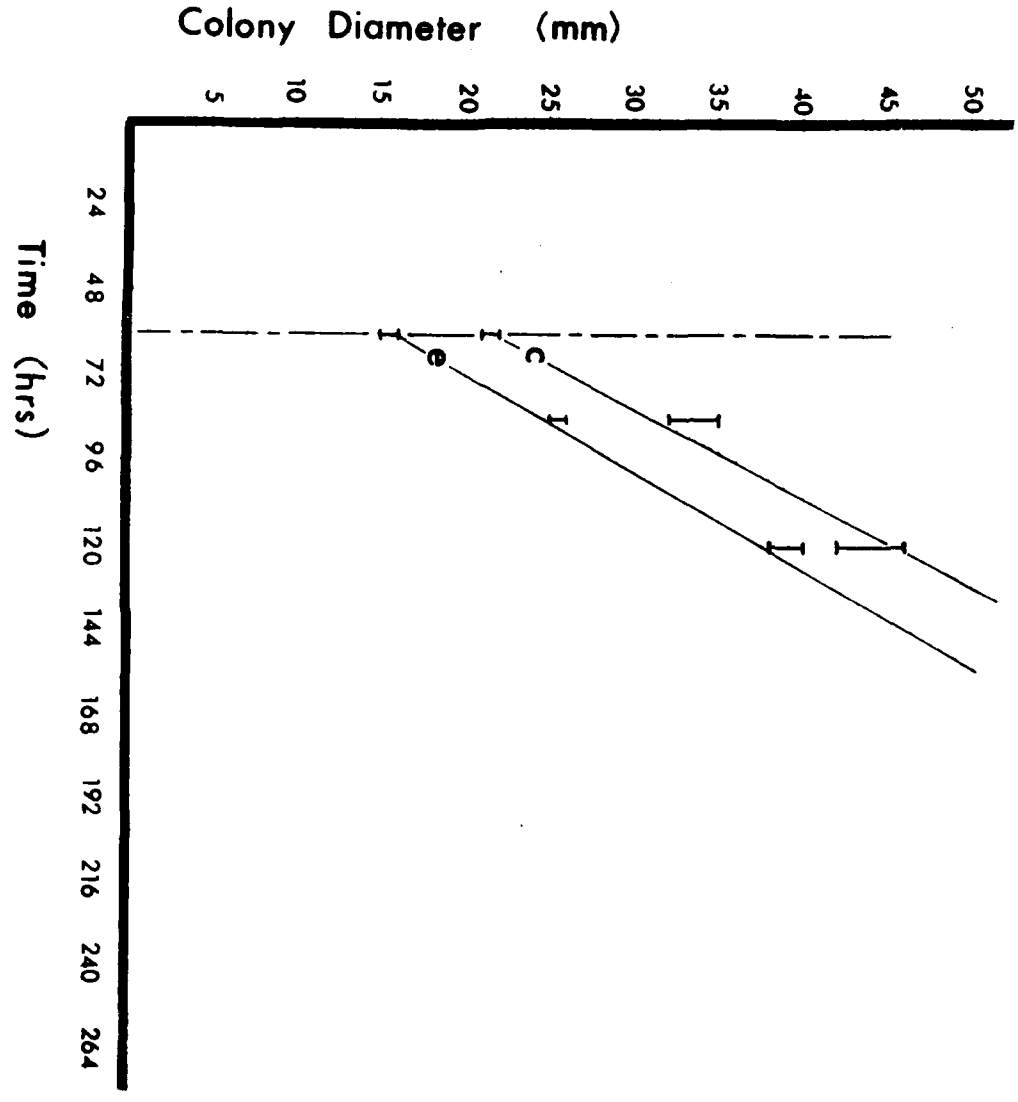
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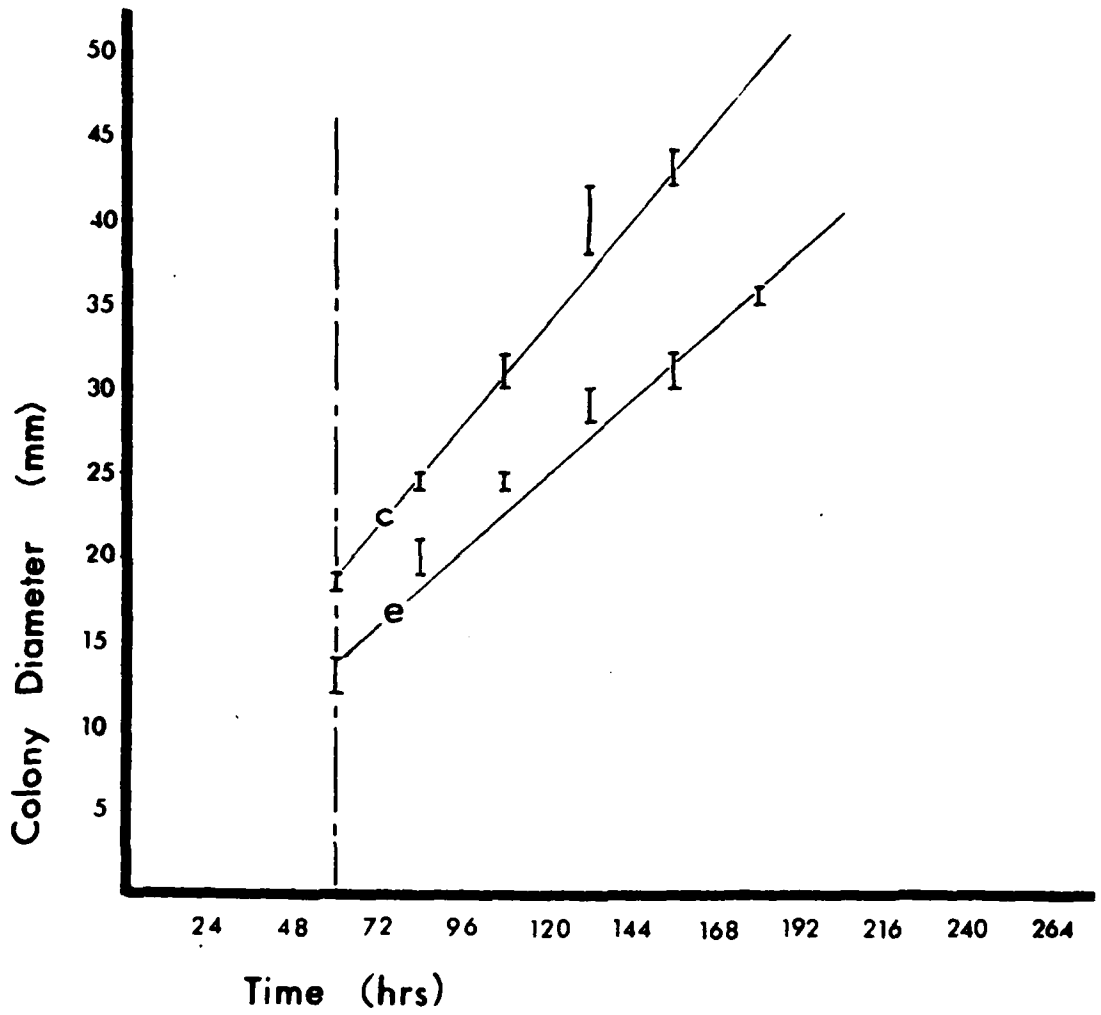
Figure 6. Growth curves for cultures of Aspergillus fumigatus subjected to "treatment table" oxygen exposures.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